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RESEARCH PAPER

Involvement of substance P in the development of cisplatin-induced acute and delayed pica in rats

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acute emesis; brain microdialysis; cisplatin; delayed emesis; medulla; neurokinin NK_1 receptor; pica; preprotachykinin-A; substance P; $5\text{-}HT_3$ receptor

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BACKGROUND AND PURPOSE
Although substance P (SP) and neurokinin NK_1 receptors have been reported to be involved in cisplatin-induced acute and delayed emesis, their precise roles remain unclear. Pica, the consumption of non-nutrient materials such as kaolin in rats, can be used as a model of nausea in humans. We investigated the time-dependent changes in cisplatin-induced pica and the involvement of SP and NK_1 receptors in this behaviour.

EXPERIMENTAL APPROACH
Rats were administered cisplatin with or without a daily injection of a $5\text{-}HT_3$ receptor antagonist (granisetron) or an NK_1 receptor antagonist (aprepitant), and kaolin intake was then monitored for 5 days. The effects of granisetron on the cisplatin-induced expression of preprotachykinin-A (PPT-A) mRNA, which encodes mainly for SP, and on SP release in the medulla, measured by *in vivo* brain microdialysis, were also investigated.

KEY RESULTS
Cisplatin induced pica within 8 h of its administration that continued for 5 days. Granisetron inhibited the acute phase (day 1), but not the delayed phase (days 2–5), of pica, whereas aprepitant abolished both phases. Within 24 h of the injection of cisplatin, PPT-A mRNA expression and SP release in the medulla were significantly increased; these findings lasted during the observation period and were inhibited by granisetron for up to 24 h.

CONCLUSIONS AND IMPLICATIONS
The profiles of cisplatin-induced pica in rats are similar to clinical findings for cisplatin-induced emesis in humans, and we showed that SP production in the medulla and activation of NK_1 receptors are involved in this cisplatin-induced pica.

Abbreviations
CMC, carboxymethylcellulose; EC, enterochromaffin; i.g., intragastric; PPT-A, preprotachykinin; RTX, resiniferatoxin; SP, substance P

Introduction

Cisplatin-based cancer chemotherapy often induces a biphasic pattern of emesis (Kris *et al.*, 1998; Roila *et al.*, 2010). Acute emesis, which occurs within 24 h following drug administration, is inhibited by $5\text{-}HT_3$ receptor antagonists such as ondansetron and granisetron. In contrast, delayed emesis, which begins 24 h after drug administration, is

resistant to 5-HT_3 receptor antagonists. To improve the anti-emetic efficacy of 5-HT_3 receptor antagonists, corticosteroids, such as dexamethasone, are frequently added and the combination regimen is increasingly used as standard therapy for cisplatin-induced acute and delayed emesis. This regimen has proved to be significantly more effective than single therapy, but some patients still experience delayed emesis despite the anti-emetic drugs and complain about the course of curative treatment (Jordan *et al.*, 2007). Substance P (SP), a neuropeptide that binds to three subtypes of neurokinin receptor, NK_1, NK_2, and NK_3 (nomenclature accords with Alexander *et al.*, 2013), is known to regulate many biological functions including respiratory rhythm, anxiety, pain and nociception via its endogenous NK_1 receptor, which it has a high degree of affinity for (Brain and Cox, 2006). Previous studies have shown that SP is also involved in the patho-aetiology of emesis, nausea, anorexia and inhibition of gastric emptying (Grønstad *et al.*, 1985; Li, 2007). In fact, chemotherapeutic agents can increase the release of not only 5-HT, but also SP from the enterochromaffin (EC) cells of gastric mucosa and brainstem (Darmani *et al.*, 2009; Dey *et al.*, 2010), and SP activates the emetic circuitry, such as the area postrema (AP) and the nucleus of the solitary tract (NTS), via peripheral and/or central neurokinin NK_1 receptors (Baude and Shigemoto, 1998). Furthermore, aprepitant, an NK_1 receptor antagonist, has been found to be effective as an anti-emetic clinically and improve the outcomes of patients receiving highly emetogenic cancer chemotherapeutic agents (Hesketh *et al.*, 2003). Tattersall *et al.* (1996) reported that NK_1 receptor antagonists act centrally to inhibit the emesis induced by cisplatin, therefore, it has been postulated that cisplatin-induced emesis is ultimately mediated by a central SP pathway. However, the precise role of SP in the central regulation of emesis remains unclear.

To investigate the underlying aetiology of emesis and to assess the anti-emetic efficacy of newly developed drugs, laboratory animals that can vomit, such as ferrets, dogs, cats and *Suncus murinus*, have been utilized in preclinical studies (Matsuki *et al.*, 1988; King, 1990; Naylor and Rudd, 1996). Rats, one of the most common laboratory animal species, have been assumed to be unsuitable for experimental studies in this field because they do not vomit (Horn *et al.*, 2013). However, it is now known that pica, which is a behaviour characterized by eating non-nutrient materials, such as charcoal, soil or clay (kaolin), can be induced in rats by several stimuli that commonly cause nausea in humans (Yamamoto *et al.*, 2002; 2004; 2007). We previously reported that both the latency and duration of pica were similar to the clinical findings for chemotherapy-induced emesis in human patients (Yamamoto *et al.*, 2011). Malik *et al.* (2007) previously reported that pretreatment with 5-HT_3 receptor and NK_1 receptor antagonist inhibited cisplatin-induced pica behaviour in rats; thus, pica can be used as a model of nausea and emetic activation. However, there are no reports on the detailed time-course analysis of cisplatin-induced acute and delayed pica in rats.

In this study, in order to investigate the role of the SP pathway in the development of cisplatin-induced acute and delayed nausea-related responses in rats, we firstly examined the time-dependent changes in cisplatin-induced pica and the effect of granisetron and aprepitant on this behaviour. Furthermore, we also investigated the time-dependent expression of preprotachykinin A (PPT-A), a precursor of SP, mRNA in the medulla of cisplatin-treated rats. Finally, we examined changes in SP release in the NTS using *in vivo* brain microdialysis.

Methods

Animals

Male Wistar rats, weighing about 250 g at the beginning of the experiment, were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were housed in home cages with α-cellulose bedding (ALPHA-dri®, Shepherd Specialty Papers. Chicago, IL, USA) in a room with a regular light/dark cycle (lights on 0600–1800 h) at a constant temperature (25 ± 1°C) and humidity (50 ± 5%). Animals were not used more than once. All experiments were approved by the Animal Care Committee of the School of Allied Health Sciences, Faculty of Medicine, Osaka University, and were conducted in accordance with the Animal Experiment Guidelines of Osaka University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Experiment 1-1: cisplatin-induced pica in rats

To determine the profile of cisplatin-induced pica and anorexia in rats, we used an automatic kaolin and food intake monitoring system (FDM700SW, Melquest, Toyama, Japan) by remodelling an apparatus for use in paired-feeding experiments (Yamamoto *et al.*, 2011). Briefly, this system consisted of an acrylic home cage (26 × 20 × 23 cm), two containers (7 × 4 × 10 cm), and a controller equipped with two load cells (weight sensor). Kaolin and food pellets (MF, Oriental Yeast, Osaka, Japan) were provided in their respective containers. Rats were adapted to the experimental environment for 7 days and allowed free access to tap water and both pellets throughout the experimental period. Kaolin and food intake were monitored every 4 h to the nearest 0.01 g, and the data were stored and analysed using a laptop PC. Kaolin pellets were prepared according to a previously reported method (Yamamoto *et al.*, 2011). Briefly, pharmaceutical-grade kaolin (hydrated aluminum silicate) was mixed with 3% (w w^{-1}) gum arabic in distilled water to form pellets similar in size to chow pellets and these pellets were then completely dried at room temperature. On the day of the experiment, rats received cisplatin (3 or 6 mg kg^{-1}, i.p.) in a volume of 6 mL kg^{-1} at 1800 h and their four-hourly kaolin and food consumption were measured on the day before and for 5 days after the injection of cisplatin. Controls were treated with saline (i.p.). There were five rats in each of the experimental groups.

Experiment 1-2: effects of the 5-HT_3 or NK_1 receptor antagonist on cisplatin-induced pica and anorexia in rats

Rats were administered granisetron (5-HT_3 receptor antagonist, 0.05 and 0.1 mg kg^{-1}, i.p.) or aprepitant [NK_1 receptor antagonist, 1 and 2 mg kg^{-1}, intragastically (i.g.)] immediately following the administration of cisplatin (6 mg kg^{-1},

i.p.). Granisetron or aprepitant was then administered every 24 h for the observation period and kaolin and food consumptions were measured. Control animals received saline (0.1 mL 100 g^{-1} body weight, i.p.) or 0.5% carboxymethylcellulose (CMC) solution (0.1 mL 100 g^{-1} body weight, i.g.) as a respective vehicle. Another group of rats were administered granisetron (0.1 mg kg^{-1}, i.p.) twice daily for 5 days after receiving cisplatin, and their kaolin and food consumptions were measured every 4 h. Control animals received saline. The scheduled injection time was set at 0, 6, 24, 30, 48, 54, 72, 78, 96 and 102 h after cisplatin administration. There were five rats in each of the experimental groups.

Experiment 2: effects of resiniferatoxin (RTX) on cisplatin-induced pica and anorexia in rats

According to Szallasi's method (Szallasi *et al.*, 1999), RTX was injected s.c. at a dose of 0.3 mg·kg^{-1} into the necks of rats anaesthetized by inhalation of 3% sevoflurane (Sevofrane®, Maruishi Pharmceutical, Osaka, Japan) followed by an injection of pentobarbital (Nembtal®, 50 mg·kg^{-1}, i.p., Dinippon Sumitomo Pharma, Osaka, Japan). Control animals were administered an equal volume of the 98% ethanol, s.c. (Sigma-Aldrich Japan, Tokyo, Japan), which was used as the solvent. Two weeks after the administration of RTX or solvent ethanol, all rats were housed in individual acrylic cages (23 × 23 × 20 cm) and food and kaolin pellets were provided in a stainless steel container placed in the respective cages. After habituation periods, rats received saline or cisplatin (6 mg·kg^{-1}) in a volume of 6 mL·kg^{-1} at the dark-phase onset time and their daily kaolin and food consumptions were measured at 24 and 48 h after the administration of cisplatin. There were four rats in each of the experimental groups.

Experiment 3-1: effects of cisplatin on the expression of PPT-A mRNA in the medulla oblongata of rats

At 12, 24, 36, 48, 72 and 120 h after administration of cisplatin at a dose of 6 mg·kg^{-1} or saline, rats were deeply anaesthetized with the inhalation of sevoflurane and pentobarbital. Brains were removed and the medulla oblongata was dissected from the surrounding tissues. Total RNA was extracted using the Total RNA Extraction System (Viogene, New Taipei, Taiwan), according to the manufacturer's instructions. RNA was converted into first-stranded cDNA with the reverse transcriptase enzyme kit (ReverTra Ace®, TOYOBO Life Science, Osaka, Japan), which was then used as a template for RT-PCR with PPT-A and GAPDH-specific primers. The sequences of primers and thermalcycler conditions for RT-PCR are listed in Table 1. PCR products were separated on 3.0% agarose gels (Nacalai Tesque, Kyoto, Japan) by electrophoresis analysis and stained with a 1/10 000 dilution of SYBR Safe (Life Technologies, Carlsbad, CA, USA). Gels were captured with E-graph (AE-900, ATTO, Tokyo, Japan) and their band densities were analysed for quantification using the ATTO CS Analyzer ver3.0. There were four rats in each of the experimental groups.

Experiment 3-2: effects of a 5-HT3 receptor antagonist on the cisplatin-induced expression of PPT-A mRNA

Experimental protocol was almost identical to that for the detection of cisplatin-induced PPT-A mRNA expression, except for i.p. administration of granisetron (0.1 mg·kg^{-1}). Granisetron was injected in the same manner as in experiment 1–2, and the brains were removed immediately or 12 h after the last administration of granisetron. Control animals received i.p. saline. There were four rats in each of the experimental groups.

Experiment 4: effects of cisplatin on SP release in the medulla oblongata of rats

Rats were anaesthetized with the inhalation of sevoflurane and an i.p. injection of sodium pentobarbital (50 mg·kg^{-1}). For the purpose of pain relief from skin incision, lidocaine (8% Xyrocaine®, 0.05 mL, Astrazeneca, Osaka, Japan) was applied topically on the skin. One end of the i.p. catheter, made of polyethylene tubing (INTRAMEDIC PE50, Becton

Table 1

List of primer sequences and thermal conditions used for RT-PCR analysis in this study

Primer		Sequence (5′-3′)	PCR products size	PCR conditions
PPT-A			220 bp	95°C for 3 min
	Sense	ACCAGATCAAGGAGGCAATG		45 cycles
				95°C for 30 s
				55°C for 30 s
	Antisense	GCCCATTAGTCCAACAAAGG		72°C for 60 s
				72°C for 5 min
GAPDH			610 bp	95°C for 3 min
	Sense	GGGTGTGAACCACGAGAAAT		25 cycles
				95°C for 30 s
				55°C for 30 s
	Antisense	TTACTCCTTGGAGGCCATGT		72°C for 60 s
				72°C for 5 min

Dickinson, Sparks, MD, USA) was inserted into the abdominal cavity and the another end was threaded s.c. to the top of the skull. Then, animals were placed in a stereotaxic apparatus (Kopf Instrument, Tujunga, CA, USA), and a guide cannula (AG-4, Eicom, Kyoto, Japan) was implanted into the NTS with the coordinates of anterior +13.7 mm, lateral +0.5 mm and ventral −4.2 mm from the bregma, according to a brain atlas of rats (Paxinos and Watson, 1998), and it was fixed with dental cement. On the day of the experiment, a microdyalysis probe with 1 mm effective length (PEP-4-1, Eicom) was implanted into the NTS through the guide cannula. According to Takeda's report (Takeda *et al.*, 2011) with a slight modification, the probe was perfused with artificial Ringer's solution (147 mM NaCl, 4 mM KCl and 2.3 mM $CaCl_2$, pH 7.4) driven by a syringe pump (ESP-32, Eicom) and a peristaltic roller pump (ERP-10, Eicom) at a rate of 1.0 μL·min^{-1}. Six hours after the perfusion, samples were collected every 2 h to take a baseline measurement and rats were then administered four sets of drug combinations, that is (i) saline and saline; (ii) saline and cisplatin (6 mg·kg^{-1}); (iii) granisetron (0.1 mg·kg^{-1}) and saline; and (iv) granisetron and cisplatin via the implanted i.p. catheter. After the injection, samples were collected for a further 36 h. SP contents in the dialysate samples were analysed by ELISA using a kit (Item Number 583751, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. There were four rats in each of the experimental groups.

Drugs

Gum arabic and kaolin were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Cisplatin (Sigma-Aldrich, St. Louis, MO, USA) and granisetron hydrochloride (Kytril® inj. Chugai Pharmaceutical, Tokyo, Japan) were dissolved in physiological saline. Aprepitant (Emend®) purchased from Ono Pharmaceutical (Osaka, Japan) was suspended in an aqueous suspension of 0.5% CMC (Sigma-Aldrich Japan). RTX (Enzo Life Sciences, Farmingdale, NY, USA) was dissolved in 98% ethanol (Sigma-Aldrich). All drugs were prepared immediately before injection. Doses are expressed as the free base.

Statisical analysis

Data are expressed as mean values ± SEM. Differences in means were analysed using the Mann–Whitney *U*-test, and ANOVA, followed by *post hoc* Dunnet multiple comparison tests, where appropriate. A P value of less than 0.05 was considered significant.

Results

Kaolin and food intake in control rats

Rats ate a small amount of kaolin on the first day of the habituation period, but none of animals ate any kaolin during the subsequent days. Control animals ate a small amount of kaolin (range 0–0.3 g) and approximately 25 g of food during the observation periods after the administration of vehicle (Figure 1A and B).

Effects of cisplatin on pica in rats

As shown in Figure 1A and B, cisplatin at a dose of 3 mg·kg^{-1} did not affect either kaolin or food consumption throughout the entire period. On the other hand, as shown in Figure 1A and B, cisplatin at a dose of 6 mg·kg^{-1} induced pica and anorexia within 8 and 12 h after administration of the drug, respectively, and these behaviours continued for 5 days. As this dose did not cause lethality during the observation period, it was selected for further experiments.

Effects of the 5-HT$_3$ and NK$_1$ receptor antagonist on cisplatin-induced pica and anorexia in rats

The pica induced within 24 h after cisplatin administration, that is the acute phase of pica, was effectively inhibited by pretreatment with granisetron (Figure 2A). Daily administration of both dose of granisetron slightly prolonged the latency of pica induced beyond 24 h after cisplatin administration, that is the delayed phase of pica, but it did not completely inhibit the cisplatin-induced increase in kaolin intake during the delayed phase (Figure 2A). Moreover, granisetron did not improve cisplatin-induced anorexia throughout the entire observation period (Figure 2B). As shown in Table 2, the administration of granisetron twice a day had no further effect on cisplatin-induced pica and anorexia in rats.

Administration of lower dose of aprepitant prolonged the latency of pica during the observation periods, but the daily kaolin consumption was comparable with the rats treated with vehicle (Figure 3A). On the other hand, treatment with higher dose of aprepitant completely abolished both phase of pica (Figure 3A). Food consumption in rats treated with both dose of aprepitant was returned to control levels throughout the entire observation period, except on the third day after the administration of the drug (Figure 3B).

Effects of RTX on cisplatin-induced pica and anorexia in rats

During the habituation periods, rats treated with solvent or RTX ate about 0.1 g of kaolin pellets and approximately 20 g of food pellets. As shown in Table 3, kaolin and food intake was significantly affected after the administration of cisplatin at a dose of 6 mg·kg^{-1} in solvent-treated rats during the observation period. However, no RTX-treated rats ate kaolin throughout the observation period. Although food consumption after the injection of cisplatin was reduced in RTX-treated rats, there were no significant differences.

Effects of cisplatin on the expression of PPT-A mRNA in the medulla oblongata of rats

Although PPT-A mRNA expression was slightly increased by the administration of cisplatin at 12 h, but these differences were not significant (Figure 4). The administration of cisplatin produced significantly higher PPT-A mRNA expression 24 h after the drug administration than that in control rats. Increased PPT-A mRNA expression was maintained throughout the observational period (Figure 4). Treatment with granisetron significantly inhibited the increased PPT-A mRNA expression in rats at 24 h only, and not at 36, 48, 72 or 120 h after the administration of cisplatin.

Effects of cisplatin on SP release in the medulla oblongata of rats

Basal SP release in the medulla of rats was about 5 pmol·L^{-1}. As shown in Figure 5, although the administration of saline

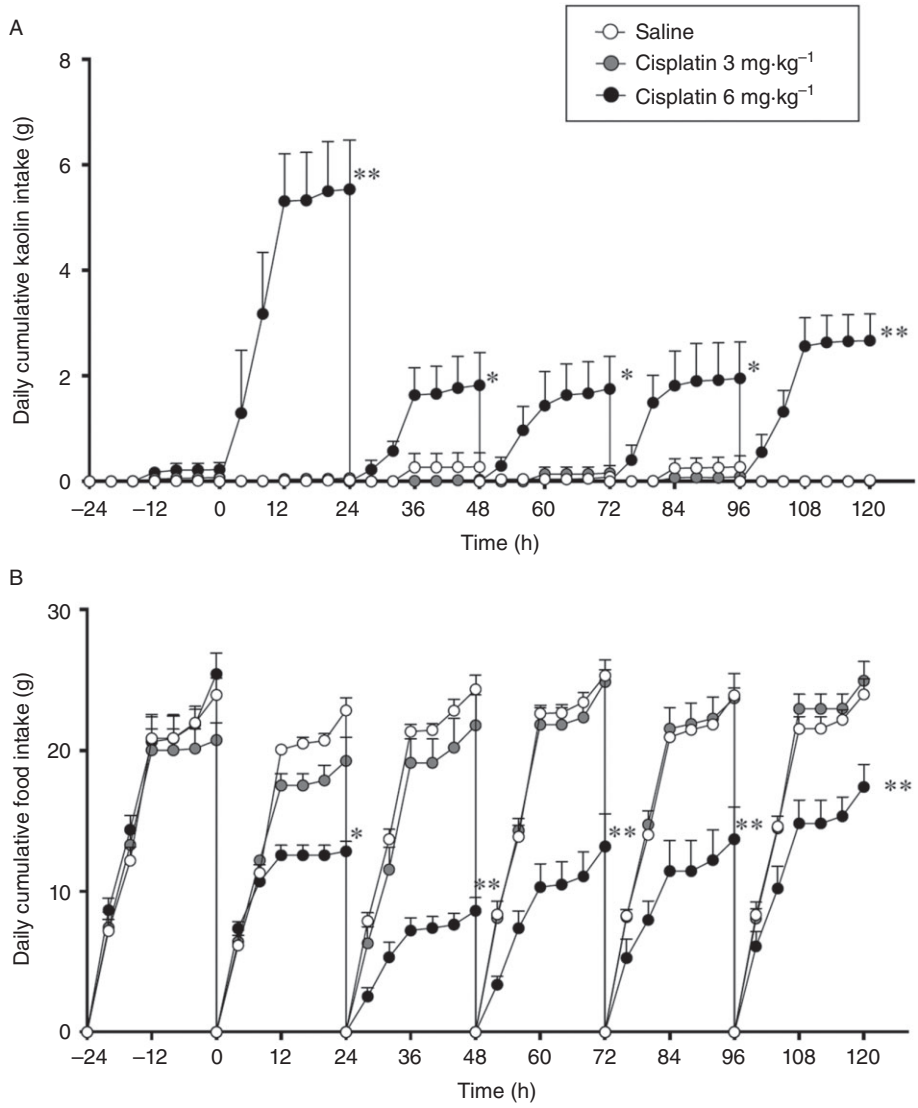

Figure 1

The effect of cisplatin on (A) kaolin and (B) food intake in rats. Cisplatin (3 and 6 mg·kg^{-1}) and saline were administered i.p. Points and bars represent the mean ± SEM of cumulative intakes every 4 h after cisplatin administration. Both intake values were set to 0 every 24 h. The data were analysed for significant differences using ANOVA, followed by *post hoc* Dunnet's multiple comparison tests. *$P < 0.05$, **$P < 0.01$ versus saline group.

or granisetron alone did not change SP release in the medulla, that of cisplatin gradually increased SP release at 12 h after the injection of cisplatin and the release induced a significant increase 16 h after the injection. The release of SP showed a continued increase during the sampling period and reached about 10 times of the basal level 36 h after the cisplatin injection. The cisplatin-induced increase in SP release in the medulla was inhibited by treatment with granisetron until 24 h after the cisplatin injection, but it was significantly elevated between 30 and 36 h after the cisplatin treatment.

Discussion and conclusion

Pica is an eating disorder characterized by the ingestion of a substance that has no nutritional value, such as charcoal, soil or clay (kaolin). The main aetiology of the behaviour has been considered to supply essential mineral substances (zinc, calcium, and iron) by eating materials (Halsted, 1968); however, a previous report suggested that pica played a role in the detoxification of toxins or elimination of gastrointestinal discomfort by ingesting soil (Johns and Duquette, 1991). We reported that pica in rats and mice was induced by emetic stimuli and inhibited by a pretreatment with the respective anti-emetic agents (Yamamoto *et al.*, 2004; 2005). Furthermore, as we demonstrated that the incidence, latency and duration of chemotherapeutic drug-induced pica are related to the clinical emetogenicity of these drugs (Yamamoto *et al.*, 2007; 2011), pica is useful for assessing the emetogenic potential or anti-emetic efficacy of newly developed drugs in preclinical studies. Previous studies have suggested that pica behaviour in rats could also be used to evaluate cisplatin-induced delayed emesis (Saeki *et al.*, 2001; Rudd *et al.*, 2002; Malik *et al.*, 2007). Clinically,

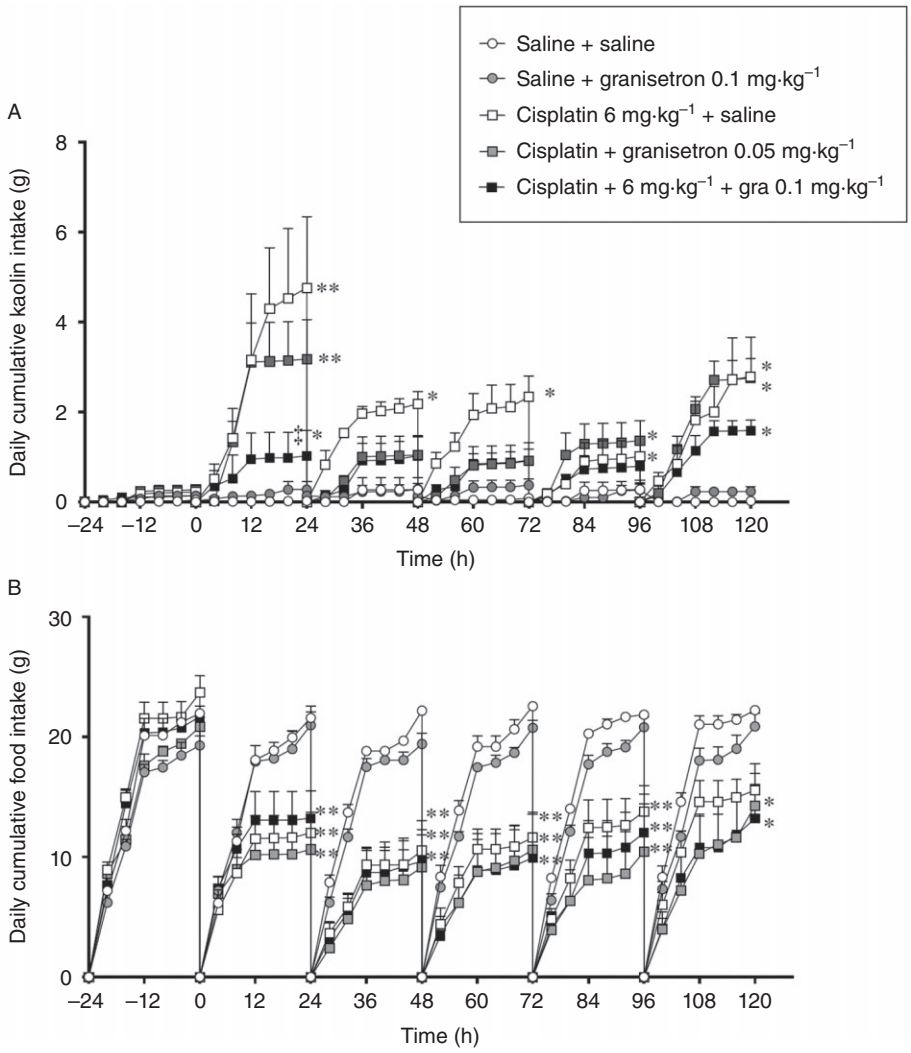

Figure 2

The effects of the 5-HT_3 receptor antagonist, granisetron, on cisplatin-induced (A) pica and (B) anorexia in rats. Granisetron (0.05 and 0.1 mg·kg^{-1}) and saline were administered i.p. every 24 h after the administration of cisplatin (0, 24, 48, 72 and 96 h after administration of cisplatin). Points and bars represent the mean ± SEM of cumulative kaolin and food intakes every 4 h after the administration of cisplatin each day. Both intake values were set to zero every 24 h. The data were analysed for significant differences using ANOVA, followed by *post hoc* Dunnet's multiple comparison tests. *P < 0.05, **P < 0.01 versus saline + saline group. ‡P < 0.01 versus cisplatin + saline group.

cisplatin-induced acute emesis occurs within 2 h and peaks at about 5–6 h following the administration of a drug (Kris *et al.*, 1998; Roila *et al.*, 2010). On the other hand, delayed emesis begins more than 24 h after drug administration. The intensity of this emesis peaks at around 48–72 h and persists for about a week. As kaolin intake was most commonly measured 24 h after receiving the emetic stimuli, the precise onset and duration of behaviour corresponding to cisplatin-induced acute and delayed emesis remain unclear. More recently, we developed an automatic kaolin intake monitoring system that was remodeled as an apparatus for use in a paired-feeding experiment, and confirmed the utility of measuring the time course of the acute phase of cisplatin-induced pica in rats (Yamamoto *et al.*, 2011). In this study, we found that the delayed phase of pica behaviour was caused as soon as the lights were turned off at 1800 h, although the acute phase of the behaviour had a latent period of approximately 4 h. The time to exhibit pica behaviour is normally restricted to the rats' dark-active phase because this behaviour is associated with feeding behaviour. From these findings, it is suggested that the time courses of the cisplatin-induced acute and delayed phases of pica in rats are similar to those of emesis in human patients. We observed the kaolin intake in rats treated with cisplatin was declined transiently on day 2, and it was increased again on day 5 (Figure 1A), because pica behaviour is also closely related to the changes of appetite in rats (Figure 1B).

The aetiology of cisplatin-induced emesis has been postulated to be that 5-HT released from the EC cells in the gut mucosa stimulates the 5-HT_3 receptor on adjacent vagal afferent nerves, which activate the central emetic circuitry in the medulla oblongata such as the AP, NTS and dorsal motor

Table 2
The effects of twice administration of granisetron on kaolin and food intakes in rats treated with cisplatin

	Pretreatment (−24 to 0 h)	Post-treatment day 1 (0–24 h)	Post-treatment day 2 (24–48 h)	Post-treatment day 3 (48–72 h)	Post-treatment day 4 (72–96 h)	Post-treatment day 5 (96–120 h)
Kaolin intake (g)						
Saline + saline (twice)	0.2 ± 0.14	0.3 ± 0.10	0.5 ± 0.08	0.3 ± 0.17	0.3 ± 0.19	0.5 ± 0.05
Saline + granisetron (twice)	0.1 ± 0.08	1.0 ± 0.63	0.8 ± 0.46	0.9 ± 0.43	0.7 ± 0.32	0.7 ± 0.25
Cisplatin + saline (twice)	0.2 ± 0.13	3.1 ± 0.97**	1.5 ± 0.47*	0.9 ± 0.29	1.3 ± 0.49*	2.7 ± 0.48**
Cisplatin + granisetron (twice)	0.0 ± 0.01	0.8 ± 0.15†	1.8 ± 0.44*	1.3 ± 0.61*	0.9 ± 0.34	1.4 ± 0.57*
Food intake (g)						
Saline + saline (twice)	23.9 ± 1.23	22.8 ± 0.88	24.3 ± 0.99	25.3 ± 1.12	23.9 ± 0.53	21.9 ± 1.11
Saline + granisetron (twice)	21.9 ± 0.43	23.4 ± 1.97	21.1 ± 2.84	21.6 ± 3.83	24.0 ± 3.59	20.3 ± 3.41
Cisplatin + saline (twice)	23.5 ± 1.73	12.0 ± 1.93**	10.5 ± 1.49**	11.6 ± 2.17**	13.7 ± 2.41**	15.5 ± 1.58*
Cisplatin + granisetron (twice)	20.4 ± 0.48	10.3 ± 1.83**	11.8 ± 1.21**	13.0 ± 1.18**	12.6 ± 1.07**	12.2 ± 0.99**

Cisplatin (6 mg·kg^{-1}) or saline were administered i.p.. Granisetron (0.1 mg·kg^{-1}, i.p.) or saline twice daily for 5 days after receiving cisplatin. Scheduled injection time of granisetron was set at 0, 6, 24, 30, 48, 54, 72, 78, 96 and 102 h after cisplatin administration. Data represent the mean ± SEM of daily intakes. The data were analysed for significant differences using ANOVA, followed by *post hoc* Dunnet's multiple comparison tests. *$P < 0.05$, **$P < 0.01$ versus saline + saline (twice) group. †$P < 0.05$ versus cisplatin + saline (twice) group.

nucleus of the vagus, and then induces an emetic reflex (Naylor and Rudd, 1996). These observations are the rationale that 5-HT$_3$ receptor antagonists, such as granisetron and ondansetron, are regarded as the first choice for the prevention and treatment of emesis (Herrstedt and Roila, 2009; Roila *et al.*, 2011). In this study, we found that granisetron completely inhibited the acute phase of cisplatin-induced pica. However, daily administration of granisetron did not completely inhibit the delayed phase of pica, although it retarded the onset time of eating kaolin pellets. Previous studies have reported that the half life of granisetron in rats is approximately 1–2 h (Huang *et al.*, 1999). Even if rats received the second administration of granisetron during the middle of dark-active phase in rats, the therapeutic effect to the delayed phase of cisplatin-induced pica remained unchanged. Similarly, clinical practice has demonstrated that many patients still experience emesis despite having received most 5-HT$_3$ receptor antagonists (Matsui *et al.*, 1996). For these reasons, 5-HT and its 5-HT$_3$ receptor are not considered to be primary mediators for the delayed phase of cisplatin-induced pica.

SP is an 11 amino acid peptide that is synthesized from PPT-A protein precursors, and it is widely distributed in the CNS and peripheral nervous systems (Ribeiro-da-Silva and Hökfelt, 2000). In the peripheral nervous system, SP is found in the terminals of primary sensory neurons and neurons intrinsic to the gastrointestinal (GI) tract, and the SP released is known to increase vagal afferent activity via adjacent NK$_1$ receptors (Andrews and Sanger, 2002). Previous studies have demonstrated that cisplatin may induce SP synthesis in the mucosa of the GI tract, and NK$_1$ receptor antagonists modulate visceral efferent functions (Greenwood-Van Meerveld *et al.*, 2003; Qian *et al.*, 2009). Furthermore, there is evidence to suggest that SP via NK$_1$ receptors enhances the release of 5-HT from EC cells (Ginap and Kilbinger, 1997). Thus, it is possible that the therapeutic effects of 5-HT$_3$ receptor antagonists on the acute phase of cisplatin-induced emesis are potentially and partially mediated by peripheral NK$_1$ receptors. In the present study, we also observed that daily administration of aprepitant, which easily penetrates the blood–brain barrier, completely inhibited not only the acute phase, but also the delayed phase of cisplatin-induced pica in rats. RTX, an ultrapotent capsaicin analogue, is known to activate the transient receptor potential vanilloid 1 on primary afferent sensory neurons, which are involved in nociception (Wong and Gavva, 2009). Previous studies have demonstrated that RTX transiently increased SP release and subsequently induced the depletion of SP levels due to a combination of neuron loss and decreased synthesis in the surviving cells (Jeftinija *et al.*, 1992; Szallasi *et al.*, 1999). Andrews *et al.* (2000) reported that subcutaneous treatment with RTX had emetic followed by anti-emetic effects in *Suncus murinus*, the house musk shrew. In our experiments, we found that RTX-treated rats did not show both acute and delayed phases of cisplatin-induced pica. From the findings mentioned earlier, we hypothesized that SP and NK$_1$ receptors act as important mediators for delayed cisplatin-induced pica in rats, but the precise role of SP is still unclear.

Previous studies have demonstrated that SP and NK$_1$ receptors are highly expressed in the preganglionic neurons of NTS, which are the terminals of vagal afferent neurons,

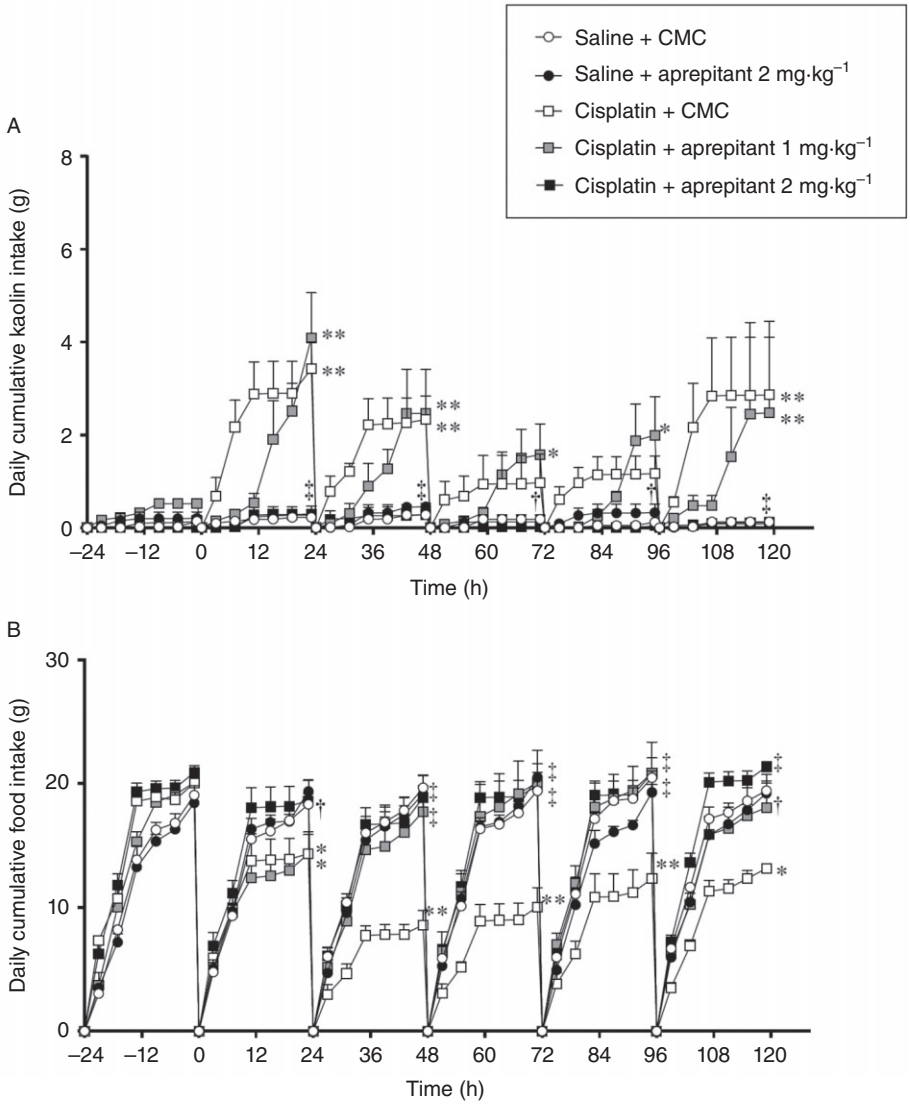

Figure 3

The effects of the NK_1 receptor antagonist, aprepitant, on cisplatin-induced (A) pica and (B) anorexia in rats. Aprepitant (1 and 2 mg·kg^{-1}) and CMC were administered i.g. every 24 h after the administration of cisplatin (0, 24, 48, 72 and 96 h). Points and bars represent the mean ± SEM of cumulative kaolin and food intakes every 4 h each day. Both intake values were set to 0 every 24 h. The data were analysed for significant differences using ANOVA, followed by *post hoc* Dunnet's multiple comparison tests. *$P < 0.05$, **$P < 0.01$ versus saline + CMC group. †$P < 0.05$, ‡$P < 0.01$ versus cisplatin + CMC group.

and that cisplatin actually increased SP-immunoreactivity in the neurons of the AP, which is located close to the NTS (Kawano and Chiba, 1984; Manaker and Rizio, 1989; Qian *et al.*, 2009). Tattersall *et al.* (1996) reported that the local administration of an NK_1 receptor antagonist into the NTS of ferrets effectively inhibited cisplatin-induced vomiting. Furthermore, Tatsushima *et al.* (2011) recently reported that the level of SP in the cerebrospinal fluid of rats was increased 72 h after an intraperitoneal injection of cisplatin. Horii *et al.* (2012) recently reported that PPT-A mRNA expression in the medulla was increased by motion stimuli that induced pica behaviour in rats. Thus, it is thought that SP in the CNS is an important mediator for the development of pica in rats through the NK_1 receptor. In this study, we also found that PPT-A mRNA in the medulla oblongata was up-regulated between 24 and 120 h after the injection of cisplatin and daily administration of granisetron suppressed the cisplatin-induced expression of PPT-A mRNA at 24 h only. The time required to increase PPT-A gene expression was comparable with the latent period of the delayed phase of cisplatin-induced pica in rats; however, from these results, the time course of cisplatin-induced SP release in the medulla remains unknown. Brain microdialysis is an analytical method that is used to determine the extracellular concentrations of various neurotransmitters in the brain of awake and freely moving animals. The strategy of our studies, therefore, was to monitor long-term dynamic changes in SP release in the medulla, especially the NTS, of rats using brain microdialysis.

Table 3

The effects of cisplatin on kaolin and food intakes in rats treated with RTX

	Pretreatment (–24 to 0 h)	Post-treatment day 1 (0–24 h)	Post-treatment day 2 (24–48 h)
Kaolin intake (g)			
Solvent-treated			
Saline	0.10 ± 0.05	0.02 ± 0.01	0.00 ± 0.00
Cisplatin	0.10 ± 0.05	5.60 ± 0.66**	2.91 ± 0.60**
RTX-treated			
Saline	0.02 ± 0.02	0.00 ± 0.00	0.01 ± 0.00
Cisplatin	0.03 ± 0.00	0.04 ± 0.03	0.02 ± 0.02
Solvent-treated			
Saline	18.9 ± 1.12	17.9 ± 0.34	18.3 ± .16
Cisplatin	19.6 ± 0.54	10.9 ± 2.01**	13.4 ± 2.85*
RTX-treated			
Saline	18.5 ± 0.36	18.9 ± 1.04	17.9 ± 1.19
Cisplatin	19.1 ± 0.50	15.3 ± 1.81	15.6 ± 2.85

Cisplatin (6 mg kg^{-1}) and saline were administered i.p. Data represent the mean ± SEM of daily intakes. The data were analysed for significant differences using the Mann–Whitney *U*-tests. *P < 0.05, **P < 0.01 versus saline group.

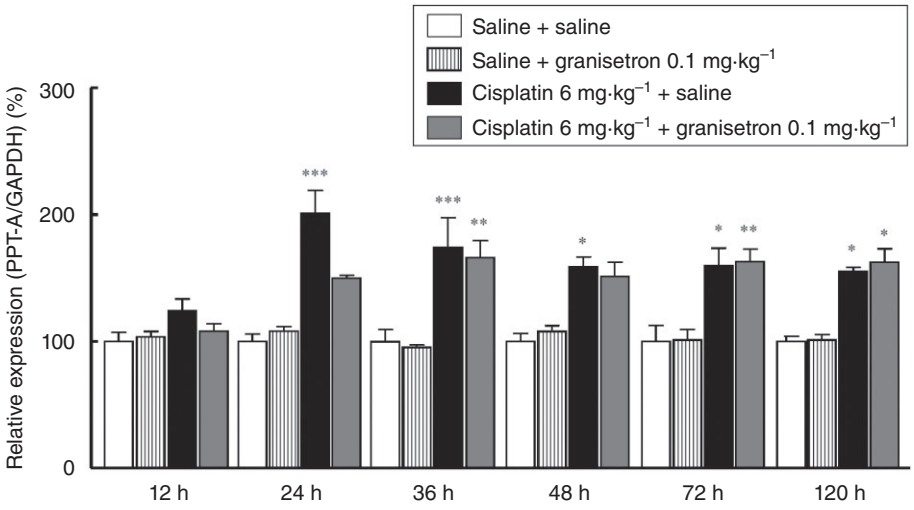

Figure 4

The effects of cisplatin (6 mg·kg^{-1}) on the expression of PPT-A mRNA in the medulla oblongata of rats treated with or without granisetron. Granisetron (0.1 mg·kg^{-1}) and saline were administered i.p. once 24, 48, 72, 96 and 120 h after administration of cisplatin. Columns and bars represent the mean ± SEM expressed as a ratio of PPT-A: GAPDH. The data were analysed for significant differences using ANOVA, followed by *post hoc* Dunnet's multiple comparison tests. *P < 0.05, **P < 0.01, and ***P < 0.001 versus saline + saline group.

The standard microdialysis probe used for *in vivo* studies normally is limited for use in recovering relatively low molecular substance compounds. However, we used a newly developed probe with a structure that allowed the recovery of large molecules such as neuropeptides and inflammatory cytokines (Takeda *et al.*, 2011). In the present study, we demonstrated that this system was able to collect ELISA-detectable levels of SP from the NTS of rats for more than 40 h. Furthermore, cisplatin treatment caused a significant increase in SP release to approximately 600% of basal levels within 18 h of its administration, and granisetron predictably antagonized cisplatin-induced increases in extracellular SP levels up to 24 h. Taken together the observation that cisplatin increased SP release and granisetron inhibited the increased SP, the pathway that 5-HT$_3$ receptor mediated SP release from the brainstem contributes to the development of the acute phase of cisplatin-induced pica in rats. Nevertheless, our results suggested that an aetiology for the increase in SP release other

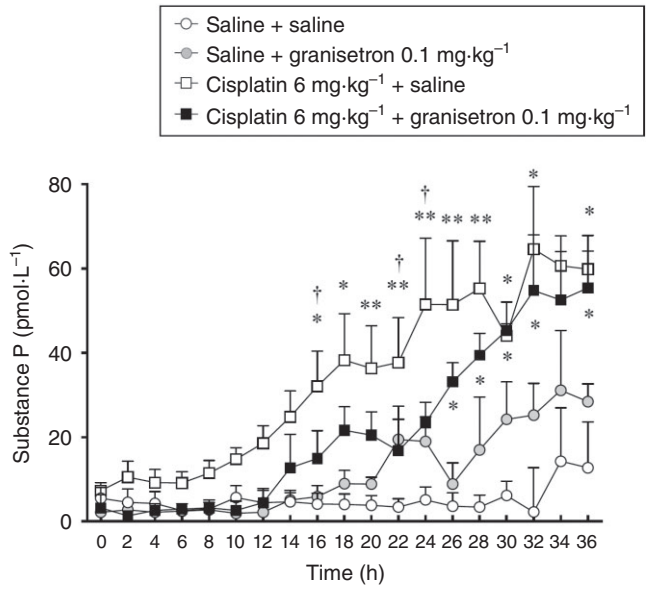

Figure 5

The effect of cisplatin on extracellular SP levels in the nucleus tractus solitarius of freely moving rats. Points and bars represent the mean ± SEM of SP concentrations. The data were analysed for significant differences using ANOVA, followed by *post hoc* Dunnet's multiple comparison tests. *$P < 0.05$, **$P < 0.01$ versus saline + saline group. †$P < 0.05$ versus cisplatin + granisetron group.

than the 5-HT_3 receptor may exist because the cisplatin-induced increase in SP release was observed even though the cisplatin-treated rats received granisetron twice in the span of 24 h. It has been reported that inflammatory cytokines such as IL-1α and β are also involved in the release of SP from sensory nerve endings and neurons (Morioka *et al.*, 2002; Skoff *et al.*, 2009). Corticosteroids, such as dexamethasone, are recommended for prophylaxis and the treatment of cisplatin-induced delayed emesis, as the potent anti-inflammatory action and reduction in the permeability of the blood–brain barrier may be involved in the anti-emetic efficacy of corticosteroids (Kovac, 2003). Tatsushima *et al.* (2011) reported that an anti-allergic agent, pemilorast, potently attenuated the release of proinflammatory cytokines and inhibited cisplatin-induced pica in rats. More recently, Darmani *et al.* (2013) demonstrated that cisplatin induced a brief increase in NK_1 receptor protein expression followed by significant increases in the phosphorylation status of the brainstem ERK1/2, which is frequently and rapidly activated by inflammatory cytokines. In addition, previous reports have suggested that this ERK1/2 pathway is necessary for modulating the expression of PPT-A and NK_1 receptor mRNA (Koh *et al.*, 2010). Therefore, these findings suggest that the production and release of SP in neurons by inflammatory cytokines also contributes to the development of the delayed phase of cisplatin-induced pica in rats.

Anorexia also occurs frequently in patients receiving cancer chemotherapy, and its precise aetiology remains unknown. We found that the administration of aprepitant, but not granisetron completely prevented cisplatin-induced anorexia in rats. Similarly, RTX-treated rats did not exhibit cisplatin-induced anorexia. Previous reports have shown that food intake was decreased by the i.c.v. administration of SP in rats (Dib, 1999) and SP modulated the hypothalamic pituitary–adrenal axis via the corticotropin releasing factor and pro-opiomelanocortin (POMC) systems, which are also known to contribute to the regulation of feeding, satiety, mood state and energy homeostasis (Jessop *et al.*, 2000; Frisch *et al.*, 2010). We reported that the activation of the POMC system in the hypothalamus may contribute to the development of chemotherapeutic agent-induced anorexia in rats (Yamamoto *et al.*, 2012). Thus, we assumed that the inhibition of SP served as a therapeutic target for the treatment of not only chemotherapy-induced emesis, but also anorexia. However, as Barrachina *et al.* (1997) demonstrated that systemic capsaicin pretreatment prevented the anorectic activity of i.p. injection of satiety substance such as leptin and cholecystokinin by selective degeneration of visceral sensory neurons, there is a possibility that this inhibitory effect of RTX on cisplatin-induced anorexia is due to peripheral afferent neurons, which is sensitive to capsaicin. Further experiments will be needed to elucidate the aetiology of this anorexia.

In summary, the time course profiles of cisplatin-induced pica in rats were similar to the clinical findings obtained for cisplatin-induced emesis in human patients, and SP production via 5-HT_3 receptors in the medulla was shown to be involved in the development of both the acute and delayed phases of cisplatin-induced pica.

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Statement of conflicts of interest

None.

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